MICROFLOW INDUCED MECHANOTRANSDUCTION IN HaCaT CELLS AND ITS APPLICATION IN CELL SHEET ENGINEERING

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By

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CERTIFICATE

This is to certify that the research project report entitled "**MICROFLOW INDUCED MECHANOTRANSDUCTION IN HaCaT CELLS AND ITS APPLICATION IN CELL SHEET ENGINEERING**" submitted by **Gautham Hari Narayana. S.N**, in partial fulfillment of the requirements for the award of the Degree of Master of Technology in Biotechnology and Medical Engineering with specialization in Biotechnology at National Institute of Technology Rourkela is an authentic work carried out by him under my supervision and guidance.

To the best of my knowledge, the matter embodied in the thesis has not been submitted to any other University/ Institute for the award of any Degree or Diploma.

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TABLE OF CONTENTS

ABSTRACT

The epidermal keratinocytes are known to elicit mechanosensitive response when exposed to fluid shear stress and often undergoes cytoskeletal reorganization and colonization. However, the exact parameters pertaining to the optimal flow rate and time of exposure for mechanotransduction is not well known. Moreover, the cellular signaling pathways involved in flow induced mechanotransduction in HaCat cells is not clear. In this regard, we used bio-microfluidic approach to evaluate to influence of flow induced shear stress on cytoskeletal reorganization of HaCaT cells (human keratinocytes) and to understand the mechanism underlying it. In present study three flow rates were experimented and 0.6 ml/h was found to be optimal. The live cell imaging of HaCaT cells under flow condition showed maximum cell spreading by 6 hours of flow exposure. So study was carried out at 0.6 ml/hour flow for 6 hours. The blocker mediated study showed that majorly actin polymerization and myosin motor protein has a crucial role in shear stress induced mechanotrasduction in HaCaT cells. Variation in lamin expression further confirmed the nuclear mechanotransduction under flow. Enhanced E-Cadherin and decreased N-Cadherin expression implied that shear stress favours epithelial phenotype. The increased expression of ZO-1, a hall mark protein for tight junction confirms that flow mediated shear stress can be used as a mechanoregulator on HaCaT cells to improve cell sheet formation therefore can enhance the permeability barrier and integrity of epidermis.

Keywords

Fluid flow, Shear stress, Epidermal keratinocytes, Cytoskeletal Reorganization, ZO-1.

List of figures

CHAPTER 1

INTRODUCTION AND REVIEW OF LITERATURE

1.1 Introduction

Tissue engineering in the late 1980's was defined as a field dealing with "the application of the principles and methods of engineering and life science for the development of biological substitutes to restore, maintain, or improve functions" [1]. In recent, advancement of tissue engineering and mechanobiology has allowed researchers to reveal many aspects of biomechanical regulation, particularly in the context of mechanical stimuli that governs the cell fate [2]. A focused study of cellular dynamics under mechanical stresses at different physiological processes and pathological conditions is regarded altogether as a field called mechanotransduction[3].

The mechanical stimulus includes forces like gravity stress, fluid shear stress, substrate stretch, cyclic strain, vibration and shockwaves that can mimic the *in vivo* conditions that cells or tissue experience. Numerous cell types are studied for their response to individual forces, the type of force utilized for the study is selected based on its ability to mimic the natural microenvironment of cells, for instance bone tissue engineering *in vitro* studies are carried out subjecting cells to oscillatory shear flow [4] and cyclic strain [5]. Similarly endothelial cell system is majorly studied for their response to shear flow [6], interestingly skin epithelial cells are recently being considered mechanosensitive and our study also 0deals with the mechanosensitiveness of HaCaT skin keratinocytes, however the responsiveness and fate of different cells depends on the magnitude and type of stimuli those respective cells undergo. Due to the response of skin epithelial cells to cyclic strain [7], matrix stiffness, the osmotic pressure these cells are yet to be explored for their functionality using shear flow as a modulator.

Of recent fluid shear is recognized as a prime mechanoregulator and it has been already reported that wall shear stress affects cellular responses like cell adhesion, migration, proliferation, remodeling of cytoskeleton and nucleus [8]. In physiological conditions, endothelial cells are exposed to shear blood flow ranging from 10-20 dynes/cm^2 and osteoblast cells experience an interstitial fluid flow ranging from 7-24 dynes/cm^2. Mechanotransduction finds use in clinical studies also for instance; the shear stress finds its relevance in inflammation, tumorogenisis and cancer metastasis[9, 10].

However the role of flow induced shear on skin keratinocytes cells has not been understood in great detail, there are only few reports published regarding perfusion culture of artificial engineered skin substitutes and again which deals on the skin in tissue level and cellular lever exploration remains unrevealed [10]. In general, the skin keratinocytes are not

exposed to fluid shear stress under natural physiological conditions and it would be controversial to venture into it without any background information. The ideology of this study is from the literature encompassing embryogenesis where the amniotic fluid volumes change linearly with the fetal size, but after keratinization at the 25^{th} week of gestation, the amniotic fluid volume and fetal size are no longer directly proportional and by the 28^{th} week the volume reaches a peak and plateaus near term gestation [11]. In conclusion, we can state that keratinocytes face a shear stress once in their lifetime which is probably during embryo maturation where the juvenile skin gets its integrity and attains an important structural aspect like permeability barrier, thus this proves to be potent mechanoregulator even in the case of keratinocytes.

1.2 Literature review:

1.2.1 Mechanotransduction: A brief outlook

Over the past two decades, the area of research of mechanobiology has attracted immense attention due to its clinical and physiological significance in almost all tissues present in the human body. Majorly studies targeted in this field aim to demonstrate – "how the cells are able to experience the various mechanical stimuli and traduce them downstream in form of biochemical signals, resulting in unique physiological response" [3, 12]. The other alternative area of detailed research in this field encompasses the approximation of clinical significance of the mechanical stresses in various pathological conditions. Numerous researches have attempted to simulate the *in vivo* physiological conditions by the specific application of mechanical stimulus such as fluid shear stress (FSS),substrate stretch also known as matrix stiffness, cyclic strain, gravity stress, vibration and shockwaves [13]. In the process of transducing these mechanical stimuli and giving rise to subsequent responses, the cells transduce the forces to the various intracellular compartments either through the diverse cytoskeletal components or by an excitable molecular unit such as a membrane receptor or protein such ion channels, instantly connected to intracellular signal transduction [14]. Since, the study of cellular dynamics under the effect of variety of mechanical stresses in differing physiological and pathological conditions is of emerging importance, this separately is now regarded to as a separate field of study collectively termed as Mechanotransduction.

1.2.2 Mechanotransduction and its application in cellular engineering

The entire process of Mechanotransduction usually comprises a complex set of a set of interactions between distinct cellular components. The increasing evidences propose the essential role of a few cellular elements such as cadherins, cytoskeleton, integrins, focal adhesion complexes, ion channels, etc. in regulating the cellular behaviour in response to the respective mechanical stimuli[15].

Primarily in the signal transduction pathway the transmembrane receptor integrins, that aid in cellular adhesion and helps in binding with the RGD peptide of the extracellular matrix. These receptors are associated with a diverse group of kinases such as focal adhesion kinases and also it is associated with adaptor proteins that including vinculin, paxillin, talin,

etc. These kinases associated with integrins further activate several downstream targets which include MAP kinases and PI3P/Akt proteins. These eventually regulate the respective cellular physiological aspects which include its proliferation, differentiation, migration, adhesion and apoptosis [16, 17].

In addition to the role of the integrins, the associated adaptor proteins play an essential role in bridging the gap between the cytoskeleton and integrins, thus governing cellular proliferation and motility [18]. However during Mechanotransduction, the mechanical stresses such as mechanical stretch, fluid flow induced shear stress and cyclic stress tends to activate the mechanical stress responding ion channels that regulate the calcium influx or efflux and thus affect the cytoskeletal remodelling and intracellular signalling [9-10].

It is essential to state that majority of the growth factors and G-protein coupled receptor driven pathways also modulate the cellular responses while subjected to diverse stress conditions, in a ligand-independent manner [19, 20]. A further investigation carried out by several researchers have identified the important role of these mechanical forces in governing the ultimate fate of the cells. A vast number of research people have identified the MSC differentiation to osteogenic, myogenic and neural cell lines upon culturing on substrates with elastic modulus also known as Young's modulus in limit of 0.1-1kPa, 8-17kPa and 34kPa [21]. For instance, Shih et al has reported that MSC response enhanced differentiation of collagen type I coated polyacrylamide hydrogel as substratum with Young's modulus of 42.1±3.2kPa in comparison to an alternative substrate with Young's modulus of 7.0±1.2kPa [20]. This study showed the importance of matrix stiffness quantified by its Young's Modulus on the fate of the stem cell.

Therefore, the matrix stiffness was confirmed to have a vital role in determining the differentiation and phenotype of MSC by repetitively related studies, for example Vittorio et al. demonstrated that PL-MSCs showed a varying response when subjected to shear stress and substrate of variable stiffness. The PL-MSC on exposure to shear stress in the limit of 6–12 dyne/cm^2 for 24 hours resulted in upregulation in expression of endothelial cell markers. In addition, when the mesenchymal stem cells on culturing to a softer matrices (3kPa stiffness) expressed Flk-1 a marker for endothelial cells, while more rigid one (>8 kPa) led to a low percentage (20%) of positive cells for this marker [22]. Similarly, the hMSC's established intense uniform stress fibers formation coupled with the up regulation in expression of myogenic and neurogenic markers of differentiation by culturing on to a nanogratings of 250

nm width of PDMS substratum [22]. Another similar study on MSCs by Nikukar et al. verified the utilization of nanoscale sinusoidal mechanotransductive protocols (10-14 nm displacements at 1 kHz frequency, which as a result induced osteoblastogenesis in the MSCs [23].

1.2.3 Mechanotransduction mediated by fluid flow induced shear stress

In recent time fluid flow induced shear stress have been recognized as a vital mechanical morphoregulator. The researches carried out in this field have tried to explore the pathophysiological significance of shear stress. However, it has already been demonstrated that wall shear stress affect mainly the cellular and also nuclear processes which include several cellular activities such as cell proliferation, reorganization of cytoskeleton, nuclear remodeling, adhesion and migration [8]. evidence of the importance of flow have been frequently recurrent, for instance such type of flow plays a vital role in the primary developmental stages of the embryo, interstitial protein transport, cell homing and recruitment during the process tissue regeneration and repair.

It is significant to state that under physiological conditions the endothelial cells are exposed to blood flow which is of higher magnitude, whereas the osteoblasts are exposed to the interstitial fluid flow of relatively lower magnitude. Specifically this fluidic flow behavior has been identified to exert a shear stress in the range of 10-20 dynes/cm^2 in the case of the endothelial cells and in the range of 7-24 dynes/cm^2 for osteoblasts, which in turn controlled the entire behaviour of the cells and eventually the fate of the respective tissue they make up.

As emerging clinical applications, the shear stress finds its relevance in cancer metastasis, tumorigenesis, and inflammation. [9]. Several research groups have identified that the fluid flow-induced shear stress significantly influences cancer cell adhesion, migration, invasive capacity, viability, and morphology [24-27]. The quintessential group of mechanoreceptors include cell adhesion molecules like Cadherins, Integrins, stress-activated or shear sensitive ion channels, autocrine receptors, glycocalyx, whereas certain components fall under the category of organellar compartments of cells like primary cilia and the nuclear membrane proteins or the DNA itself which might undergo conformational changes under the influence of fluid flow, therefore affecting the global gene expression. These conformational

changes in DNA comprise majorly of the following two epigenetic phenomena Histone acetylation or deacetylation and DNA methylation or demethylation. [28].

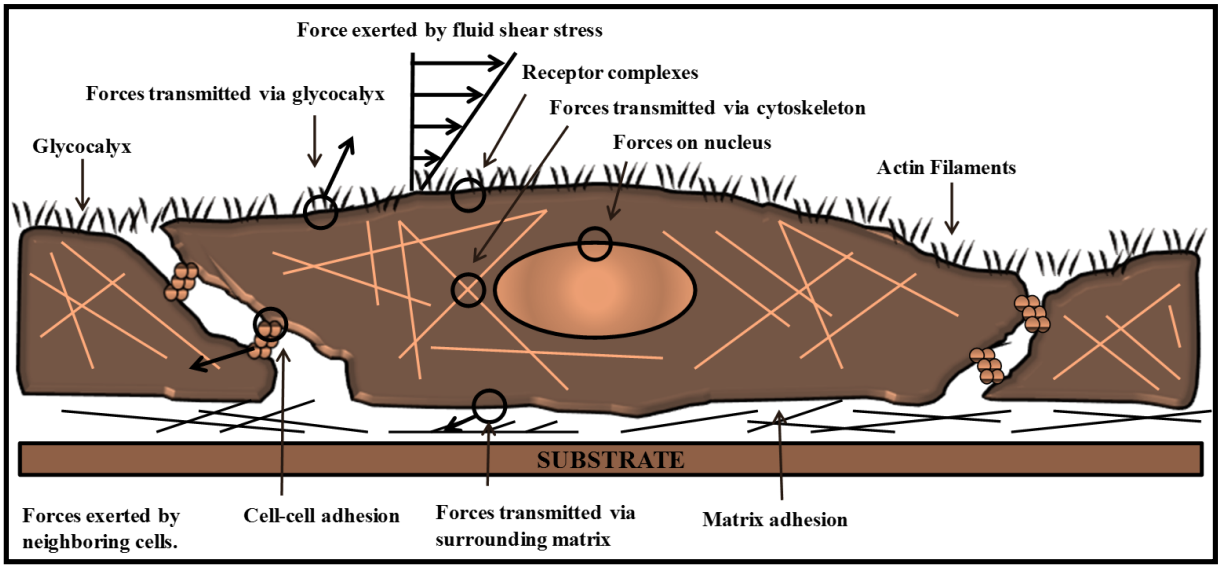


Figure.1 A schematic representation of all the mechanoreceptors in a cell being exposed to fluid flow induced shear stress and the transduction of mechanical forces into the cell.

1.2.4 Microfluidics system: An innovative tool for studying the influence of flow on cell physiology

The area of microfluidics deals with fluid volume in the nanoliter volume range through accurately designed microchannels with at least one of its dimensions in the micrometer range [29]. Interestingly, the microfluidic systems have a list of merits over the conventional macro-order systems which include the need for small volume of liquid analytes for the distinctive assays, decreased reaction time, providing high throughput, and increased sensitivity due to enhanced surface area to volume ratio[30]. The emergence of the field of microfluidics had been a revolutionary landmark in the area of molecular biology, bioengineering, and biochemistry. The applicability and relevance of the microfluidic systems in the above-mentioned area of research made it a novel realm of research coined as Biomicrofluidics [31]. In certain scenarios, on the basis of specific needs, even individual parts of a single cell can be chemically or physically manipulated through microfluidics appropriately [32]. This kind of precise functionality permitted the utilization of microfluidic systems in fundamental cell biology and associated medical diagnosis [33].

For microfluidic perfusion culture in a conserved parallel plate flow chamber, the resulting parabolic flow profile yields the wall shear stress that is given by[34]:

$$\tau = 6\mu Q/h2w$$

Where, **μ** is fluid dynamic viscosity, **w** is flow channel width, **Q** is flow rate, and **h** is channel height. In this regard, the Reynolds number (Re) can be used to determine whether the flow is laminar or turbulent. For parallel plate flow, is defined as[34]:

$$Re = \rho Q/\mu w$$

Where, **Q** is flow rate, ρ is the media density , **w** are flow channel width and **μ** is fluid dynamic viscosity.

1.2.5 The skin and its cellular organization:

Skin, the largest organ in the human body has an area of about 20 ft^2. It has a diverse array of utilities which include protection from chemical, physical and biological hazards which includes body temperature, microbes and permitting the sensations of touch, heat and cold. For majority of mammalian species, the skin is composed of three primary layers namely: 1) the outermost skin layer which behaves as a barrier denoted as the epidermis; 2) dermis lies beneath the epidermis that is composed of sweat glands, connective tissues and hair follicles which act as a cushion against any kind of external stress or strain; 3) the deeper layer comprises of the subcutaneous tissue made of fat and connective tissue, hypodermis. It is observed that the dermis is majorly comprised of fibroblasts that control the organization of the fibrillar dermal matrix; whereas the epidermis consists of keratinocytes. The skin contains five distinct layers including stratum basale, stratum spinosum, stratum granulosum, stratum licidum and stratum corneum (respectively from base to top). The stratum basale is majorly composed of basal keratinocytes that are considered to generally stem cells of the epidermis. The cells tend to move up; in the process the cells experience differentiation and eventually become anucleated. During this process, the keratinocytes organize themselves; this forms adherent junctions, secrete keratin protein and lipids which comprise essential elements of ECM and contributes to its overall mechanical strength [35].

1.2.6 Shear stress and skin keratinocyte cells: A brief overview

It has been hypothesized yet again always been a controversy to study the skin keratinocytes response with corresponding exposure to fluid flow. This is due to the fact that in natural physiological condition fluid mediated shear stress has no role in skin and its

structure. However certain studies on perfusion reactor system have been carried out to maintain the skin graft tissue and continuously nourishing it through the continuous flow of medium has been carried out [36]. These perfusion studies have been majorly carried out on fully developed skin grafts and thus this field remains unexplored to the cellular level response of skin keratinocytes to flow characteristics.

Interestingly thinking back about embryonic stages of fetal development, a study in nature article states about amniotic fluid and fetal development , in that study they have proved that keratinization process of fetal skin begins at 19^{th} week of gestation and is usually completed by 25^{th} week after implantation of zygote in the womb endometrium. When the keratinization process is complete, the correlation between the fetal size and respective amniotic fluid volume is no longer a linear relationship all of a sudden and by the 28^{th} week of gestation, amniotic fluid volume becomes doubled and then it plateaus near the terminal gestation period and thereafter finally decreases at 42^{nd} week [11]. This sudden increase in amniotic fluid volume after keratinization of skin in fetal stage gives an idea that the skin cells are exposed to shear stress due to fluid at some point of a fetal life and that time when it is exposed is the vital time where it attains its vital properties like permeability factor and tightly integrated skin structure.

1.2.7 Rationale of the study:

Over the current years, most of the researches has invoved in interpreting the effect of mechanical stimulli on the skin organization and its remodeling particularly in significant wound healing and repairing [37]. The phenomenon of the wound healing majorly involves the proliferation, differentiation and migration of the epidermal keratinocytes, therefore making this the epicenter for analyzing effects of these mechanical stresses. However, it has previously been identified that the mechanical stimulli induces the corresponding skin cell differentiation, whereas the application of mechanical stretching triggers the keratinocyte proliferation and growth [37-39]. Therefore, it is necessary to mention that the cellular behavior under the influence of different mechanical stresses such as matrix stiffness, mechanical stretch, cyclic strain follow the integrin mediated signal transduction pathway. Though, none of the prior literature indicates the effect of shear stress especially on epidermal keratinocytes. Interestingly, the fluid flow induced shear stress is considered as a vital mechano- regulator and is known to modulate the behavior of various cell types including muscle cell, endothelial cells, fibroblasts, osteoblasts [40-43].

In the recent years, several research groups have demonstrated that fluid flow-induced shear stress is capable of stimulating the cellular response in the renal epithelial and biliary cells [44, 45]. Incidentally, it is rather intriguing to discover the influence of shear stress on the epidermal keratinocyte behavior. Though, these cells never affect the fluid flow, yet the flow-induced shear stress could be used for specialized applications in cellular engineering.

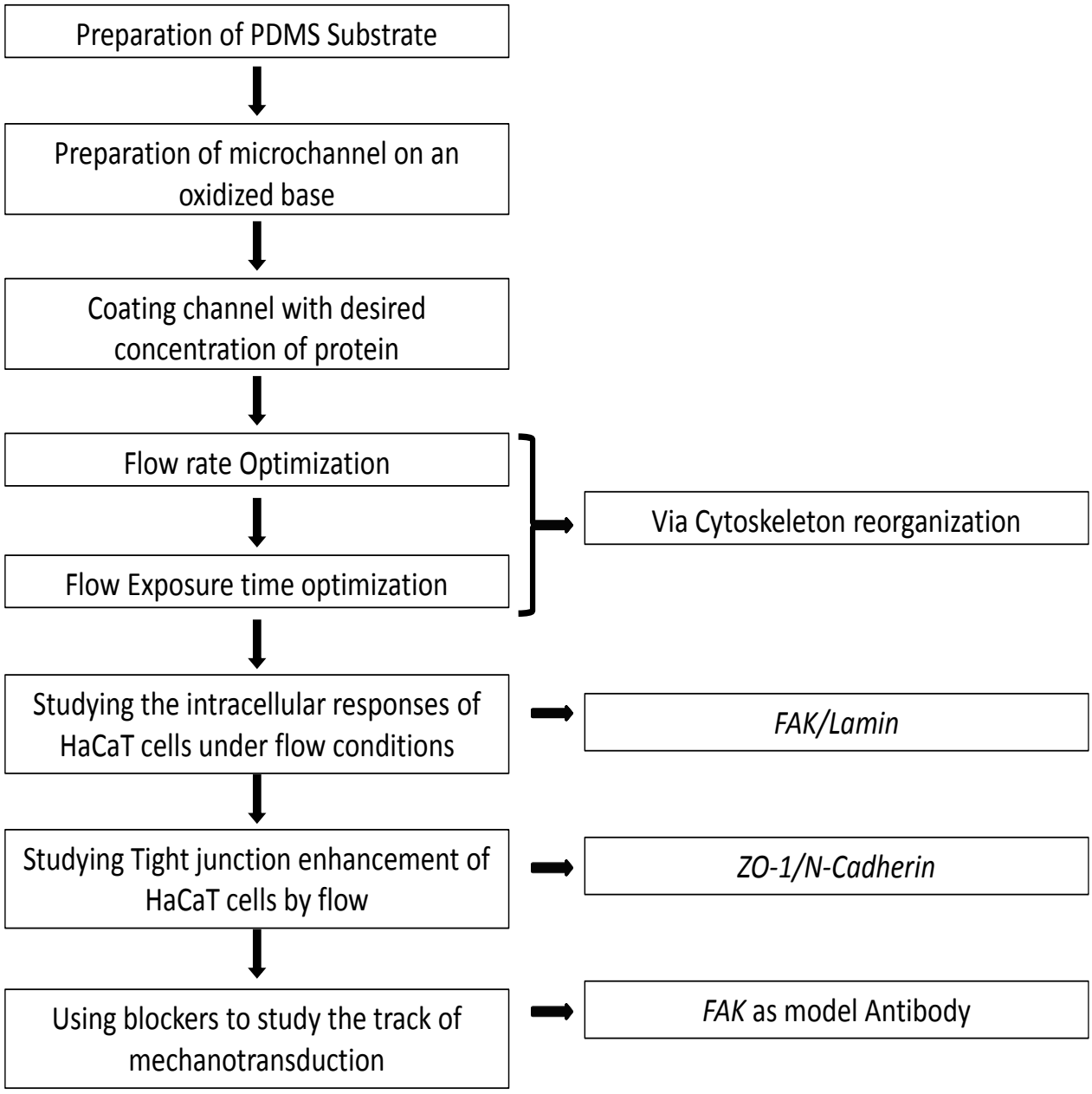
1.3 Objectives of the study:

- To study the role of fluid flow on cytoskeleton reorganization and cellular signaling in HaCaT cells.

- To decipher the molecular signaling pathways involved in flow induced mechanotransduction in HaCaT cell.

- Fluid flow induced improvement of cell sheet formation in HaCaT cell sheet for skin tissue engineering.

1.4 Work plan of the study:



CHAPTER 2

MATERIALS & METHODS

2.1 Materials:

HaCaT human Keratinocyte cell line was purchased from NCCS (Pune). PDMS (Polydimethylsiloxane) was procured from Dow Corning. DPBS (Dulbecco's Phosphate Buffer Saline), DMEM (Dulbecco's Modified Eagle's Media), Antimycotic-Antibiotic solution, Trypsin-EDTA solution were procured from Himedia, Mumbai, India and FBS (Fetal Bovine Serum) was obtained from Gibco. FAK, Lamin, N-cadherin, E-cadherin, ZO1 antibodies were brought from Abcam. DAPI and TRITC-Phalloidin were procured from Sigma-Aldrich, India. For pumping needs syringe pump (model: SP-1000) was purchased from Ningbo Annol Medical Device Technology Co. Ltd.

2.2 Methodology:

2.2.1 Preparation of Microfluidic Channel and development of microfluidic system setup for the entire study:

The microfluidic system was made using a combination of the cross linker (sylgard) and Polydimethylsiloxane (PDMS) in 1:10 (w/w) ratio. The moulds were filled by decanting crosslinked PDMS into it, desiccated , fabricated with elevated temperature of 70 °C for a duration of 120 minutes in a Hot air oven (Labotech, India) [46]. Following which, the crosslinked PDMS of the microchannel dimensions was cut out of the negative mould with specific dimensions and cleaned with 70% Isopropanol. A glass slide base is used for the microchannel which was oxidized with the aid of Piranha solution (H_2O_2 and concentrated H_2SO_4 in 1:1 (v/v) ratio) prior to fabrication. The fabricated crosslinked polymer of required microchannel dimensions was pierced for outlet and inlet using a blunt-end needle (20 gauges), a thin PDMS layer was creamed on base of microchannel and gently press-bound onto the surface of the oxidized glass cover slips carefully to prevent cracking of the base glass slide. Upon securely pasting the crosslinked PDMS microchannel it is heated up to a temperature of 70^oC for stable adhesion as a result of PDMS polymerization. Later the channel glass base was coated with three different concentrations of gelatine protein (1%, 2%, and 3%) to enhance the cell adhesion and incubated for 3 hours at 37^oC in a humidified chamber. Then the optimum concentration for homogenous uniform coating on base was verified by adding Bradford

reagent into the channel after protein coating incubation time and then viewed under a light microscope.

The microfluidic assembly was done by connecting the pump tubings to the outlet and inlet wells of the microchannel and eventually connecting the microchannel inlet port tubing to the syringe pump needle apparatus. The microfluidic channel system and syringe pump setup was utilized for both continuous flows and live cell imaging purposes. During the continuous flow exposure time period, the complete system was placed in a CO_2 incubator with 5%CO_2, at 95% humidity and 37ºC. Phase contrast microscope (ZEISS PrimoVert) was used for live cell imaging connected to a computer system for simultaneous recording.

2.2.2 Micro confinement of HaCaT Cell Culture:

HaCaT human Keratinocyte cells were obtained from NCCS (Pune, India). The cell line was cultured in DMEM (HiMedia) supplemented with 10% FBS (GIBCO) along with 1% antibiotic and antimycotic solution (HiMedia) in a CO_2 at 37ºC and at 95% humidity. The confluent cells were suspended with the help of 0.25% Trypsin-EDTA solution (Himedia) and using Trypan blue dye exclusion assay cell viability was estimated. Thereafter, the oxidized channel glass base was coated with optimized 2% gelatine and placed in the CO_2 with the above pre-set conditions for 3-4 hours and then 10µl ($1x10^6$cells/ml) of the harvested cell suspension post DMEM neutralization and centrifugation was inoculated into the microchannel system via the inlet port. Both the inlet and outlet ports were filled with complete media to prevent complete dehydration of the channel. The cell seeded microchannel systems were placed in the CO_2 incubator at 37ºC, 5% CO_2 and 95% humidity for 16-18 hours to assure all the cells adhere to the base of the channel. Then the microfluidic assembly was done by connecting tubing to the outlet and inlet wells of the microchannel and finally connecting the system to the syringe pump needle setup. The microfluidic and syringe pump setup was utilized to study the property of flow induced shear stress on HaCaT cells for 6 hours. The whole setup was placed in an incubator at 37ºC and 95% humidity. Phase contrast microscope (ZEISS PrimoVert) was used for snap imaging and also live cell imaging.

2.2.3 Flow rate and exposure time optimization:

The adhered cells on the glass slide base of the microchannel setup was exposed to the fluid flow induced shear stress with the help of a syringe pump (model: SP-1000, Ningbo Annol Medical Device Technology Co. Ltd.) continuously pumping DMEM medium. Primarily, the variation in cellular morphology was noted as a function of fluid flow rate into the microfluidic system for around 2 hours. A continuous flow (0.6ml/hr, 6.0ml/hr and 60ml/hr) was applied in the microchannel placed in an incubator at 37ºC, 5% CO_2 and 95% humidity. Then the cells exposed different flow rates were immunostained with TRITC Phalloidin and DAPI.

Briefly, the cells cultured in the microchannel system were rinsed with PBS (pH 7.4), treated with 4% Paraformaldehyde for 15 minutes for proper fixation and washed again with PBS. The rinsed cells were next permeabilized for 15 minutes with 0.1% Triton X-100 in PBS. Permeabilization is followed by immunostaining with DAPI (1:1000) and TRITC Phalloidin (1:300). The immunostained cells were then viewed under a confocal microscope to check their difference in the cytoskeleton. Finally, the optimized flow rate was used, and cellular morphological variation were analyzed as a function of time for around 8 hours using live imaging in Phase Contrast Microscopy (ZEISS PrimoVert) at 10X magnification

2.2.4 Flow modulated cytoskeletal and nuclear remodeling:

To study the resulting nuclear and cytoskeletal reorganization, of the cells which were exposed to optimum flow rate of 0.6 ml/hr for time period of 6 hours and the whole flow setup was kept into a 37ºC, 95% humid incubator. HEPES buffer was added to the medium used for flow to overcome the CO_2 deficiency of incubator and as a control system the cells were inoculated into the microchannel system and placed into 37ºC, 5%CO_2, 95% humid incubator and denoted as static control. Then the cells of both static and flow system were stained with DAPI and TRITC Phalloidin. Nuclear and cellular spreading area and their respective elongation factor were evaluated using confocal images (Leica) using MBF ImageJ software. To lessen the chances of any ambiguity regarding the significance of the assay approximately 30 cells (3-4 confocal images) were taken for the analysis.

2.2.5 Optimization of Blockers studies using FAK as model:

A future prospect to find cellular and nuclear mechanotransduction governing key pathways:

To find the track of cellular mechanotransduction through the cell cytoskeleton and its importance in cellular signals four major blockers were procured from Sigma-Aldrich which include 1) Cytochalasin D that binds to the barbed ends, i.e. fast growing plus ends of microfilaments, inhibiting actin monomer assembly and disassembly [47], 2) Blebbistatin which is known to bind with Myosin-ADP-Pi with high affinity and correspondingly interfering with phosphate release process [48], 3)Methyl β-Cyclodextrin removes cholesterol from cultured cells and also effectively disrupt lipid rafts [49], 4)Mono-ethanolate blocks MEK which in turn inhibits activation of MAPK (ERK 1/2) by the inhibition of the kinase activity of MAP Kinase Kinase (MAPKK or MEK 1/2) [49].

2.2.6 Immunocytochemistry (ICC):

To carry out Immunocytochemistry, after the adhesion of the seeded cells they were exposed to flow within the cell adhered microchannel as prescribed formerly. Then the shear stress exposed cells in to the channel were fixed with 4% Paraformaldehyde in PBS (pH 7.4) for 15 minutes, followed by 0.25% Triton X permeabilization in PBS for 10 minutes. Thereafter, blockers were added with 1% Bovine serum albumin and 0.3M Glycine in PBS, the cells were then incubated in a humid chamber at 95% humidity with the respective primary antibodies for 1 hour at 37^{o}C. The study of immunoreactivity was done by utilizing appropriate primary recognizing fluorophore-conjugated secondary antibodies using confocal microscopy (Leica system).

2.2.7 Statistical Analysis:

Corresponding Image processing and analysis were done utilizing MBF ImageJ. The co-localization analysis was represented in terms of ICQ (intensity correlation quotient) box plots.

CHAPTER 3

RESULTS & DISCUSSION

3.1 Fabrication of Microfluidic Channel system:

Using soft lithography technique, a mould was developed on a glass slide coated with a photosensitive polymer. In brief, a light sensitive polymer is coating is done on a solid glass slide. On exposure to high-intensity ultraviolet light the photosensitive polymer, it hardens (in case of negative photoresist usually SU8 photoresist) [50], the UV light is passed through a slit photomask and few parts of it is darkened (desired microchannel shape). The un-polymerized photosensitive coating was removed by a particular developing solution; the solidified photoresist fraction is used as a negative mold. Finally, these patterns were the negative mimic of channel fabricated on it.

Polydimethylsiloxane (PDMS) was used for the preparation of the substrate. Dimethylsiloxane monomers and oligomers were mixed with polymerization catalyst. Decanting the polymerized mix into the mould and incubating it at an elevated temperature, the complete fabricated channel was prepared. The mold provides the shape, grove and frame to the PDMS poured into it (figure 2). Subsequently, the substrate was peeled off the mold and it was surface sterilized with 70% ethanol (figure 3), to make inlet and outlet holes in channel, the channel reservoir ends were pierced a 20 gauge laser cut blunt end needle. Further, the prepared microchannel was laid on to the glass coverslip and baked at 70°C for better covalent bonding (figure 3).

The glass coverslip which was used as a base was oxidized wit Piranha solution (this makes the surface of the base; hydrophilic and its suitable for bonding of the protein used for coating). Further, the activated substrate was coated with three different concentration of gelatine (1%, 2% and 3%). It was observed that 2% gelatine coating resulted in a complete protein coated oxidized glass base using bright field microscope and above the concentration 3% gelatine coat didn't show much difference from that of 2% coating showing the saturated coating concentration (figure 4). This completely fabricated microchannel setup was then used for cell culturing and flow induced shear stress study on HaCaT cells.

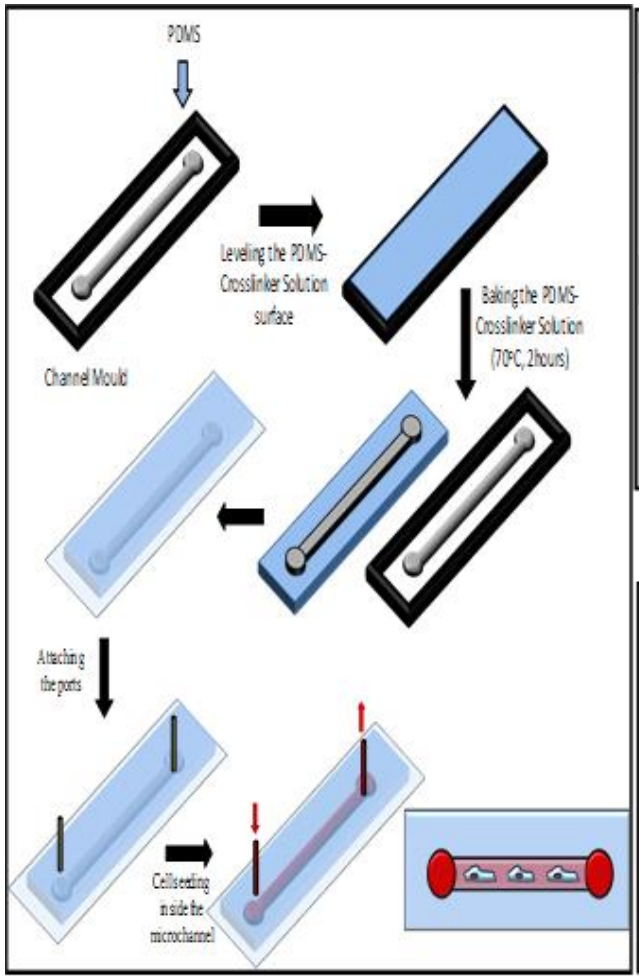


Fig 2: Schematics of Microchannel fabrication and HaCaT seeding

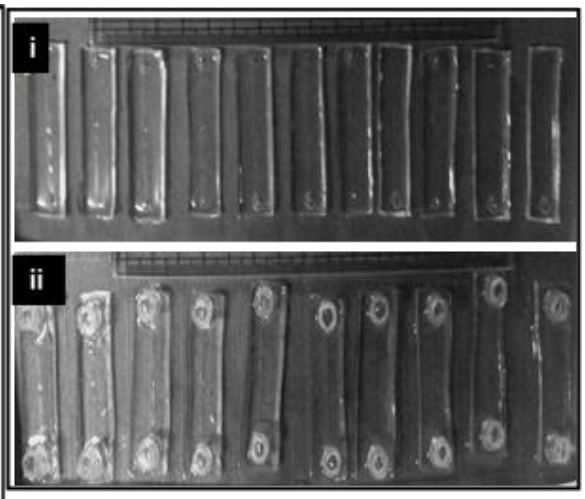


Fig 3: (i). Prepared PDMS substrate (ii). Completely fabricated

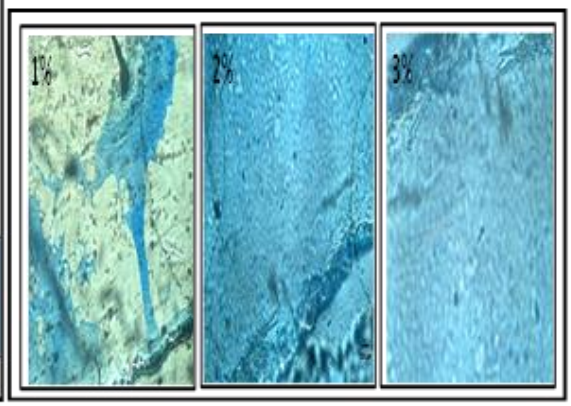


Fig 4: Gelatine coated microchannel of varying concentration

3.2 Cell Culture in Microfluidic channel:

In comparison with the macro- system, culturing and maintenance of cell lines with in a microchannel requires a keen attention. One vital reason is the nutritional constraint in the microchannel. Owing to a small volume in the microchannel (<10µl), the micro confinement, limits the volume of media accessible for the cells, and quick depletion of media supplied to microchannel was observed. Moreover, the absence of diffusive dilution results in the accumulation of the toxic metabolites which further leads to unfavorable cell culture conditions. To avoid such conditions, a fed-batch process was preferred to replenish the media at regular time interval.

The interval time for replenishment of media was decided depending upon the concentrations of cell inside the microchannel. In our analysis, the channel were seeded with a cell concentration of 10^5cells/ml and if there was a requirement to maintain microchannel with cells for longer time the channel after well adhesion of cells the channels would require a media

replenishment at every 4-5 hour after the well adhesion of cells. Prior to the seeding of cells, the microchannel was coated with gelatine (20mg/ml) to enhance cell adhesion. For proper adhesion of cells, it was incubated in a humidified CO_2 incubator for 12-16 hours. From that point forward, the cell-seeded into microchannel were exposed to shear stress induced by fluid flow. Earlier in cell culture, the microchannel sterility also becomes a chief issue. The sterility of the channel was conserved by autoclaving at 15psi, 121^{o}C for 20 minutes with PBS filled within the microchannel and reservoir wells. Further, the channels were UV and ethanol sterilized.

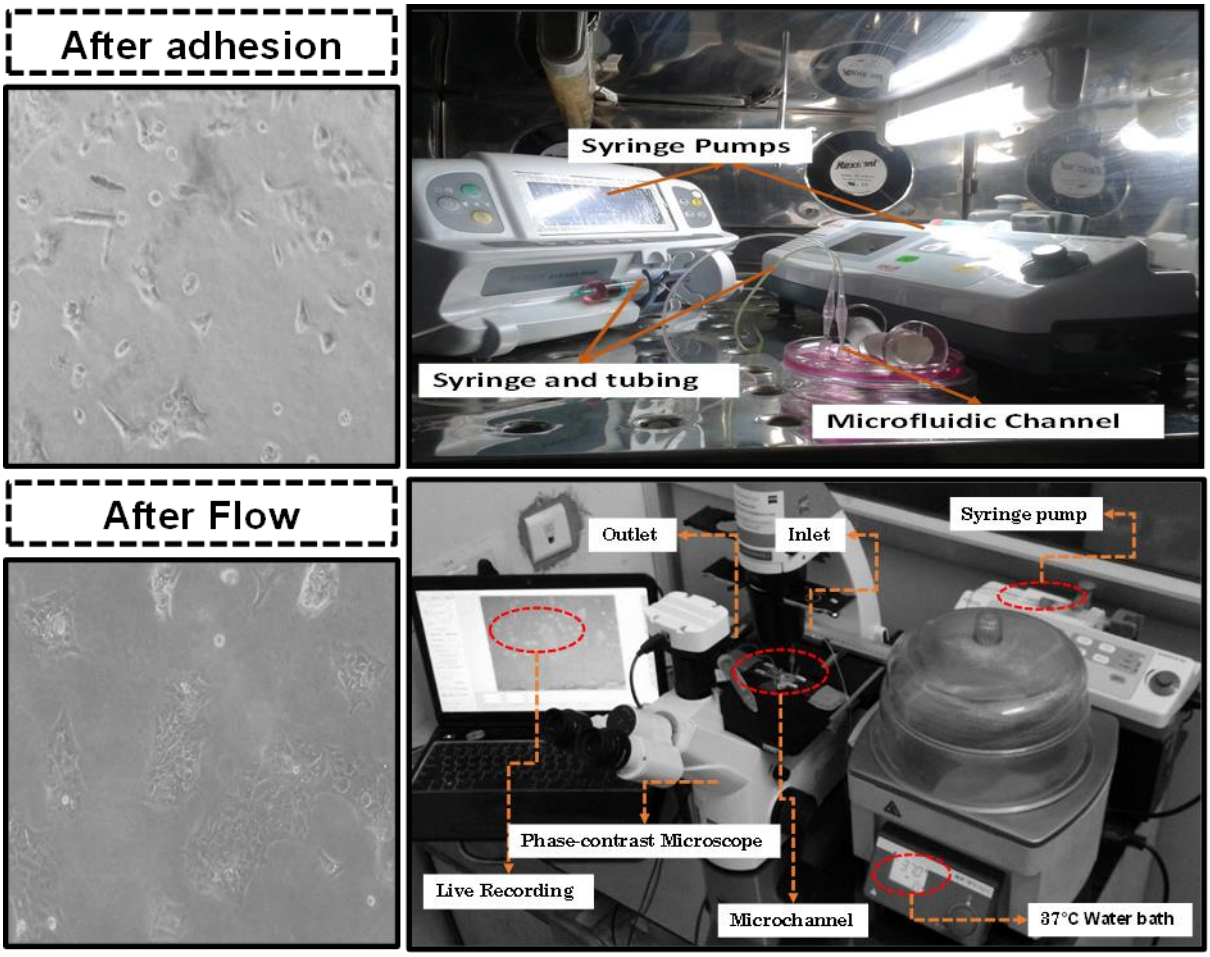


Figure 5: The arrangement of microchannel during flow mediated cell study.
A: Flow set up made for the channel incubated at 37°C, B: The flow set up made for real time live cell imaging during flow condition.

For proper cell survival, residual alcohol inside the microchannel was rinsed by washing the channels thrice using PBS, pH 7.4 or serum-free incomplete medium.

In order to deliver a continuous flow with in the channel, it was coupled to a syringe pump via connecting tubes. At different flow rates, the fluid flow inside the microchannel was optimized. The optimization of the flow was done by analyzing the volume of fluid passing

through the microchannel per minute. From that point forward, the actual flow rate was related with the theoretical flow rate (volume/unit time). The microchannel with HaCaT cells seeded to it was connected to inlet and outlet tubing and placed in 37°C incubator and media used for flow was provided with 25mM HEPES to subside the CO_2 deficiency (figure 5A). For live cell imaging the channel was placed on the phase contrast microscope and then the flow was given from inlet port to overcome the temperature fluctuation related issues the majority of the inlet tubing was immersed into the 37°C water bath and then the flow was maintained (figure 5B)

3.3 Optimization of flow rate and time of exposure for HaCaT cells cultured in microchannel:

Widely the reports about the physiology of various types of cells that experience the mechanical stimulus due to flow in *vivo* conditions, states that shear stress of wide range 7-24 dynes/cm^2 is experienced by various types of cell in body. But in regard to skin keratinocyte cells there are only a few studies on perfusion reactor based methods for artificial skin substitutes. Those study states that at lower flow rates cell viability and epidermal barrier function for grafting artificial skin was reliable, whereas higher flow rates lead to deterioration of engineered skin substitutes. In our research we concentrated on three different flow rates namely 0.06, 0.6 and 6 dynes/$cm^{2,}$ these test parameters were selected based literature views. The idea was that the skin cells respond to fluid shear stress but only at low flow rates and therefore all three test flow rate were selected with caution that it stays within the low shear stress range in comparison with natural physiologically reported values. Our confocal microscopy results and the phase contrast view of cell morphology deciphers that at low flow shear of 0.06dynes/cm^2 (0.6 ml/Hr), the HaCaT cell colony morphology and actin stress fibres showed very good integrity compared to the static no flow system (figure 6A).

Interestingly this flow rate also lies parallel to the idea discussed earlier about amniotic fluid, there it was said that in a time period of three weeks after keratinization the volume of fluid reaches a peak from 400 to 800ml and then slowly reaches plateau (roughly calculated volume will be 0.79 ml/hr) which shows the significance of results obtained that at very less shear rate due to continuous low volume change of amniotic fluid is vital for skin development as the good spreading we observe inside the channel at very low shear stress of 0.06 dynes/cm^2

on HaCaT cells . Therefore, the flow rate of 0.06 dynes/cm^2 was taken as study parameter out of three test parameters 0.06, 0.6 and 6 dynes/cm^2.

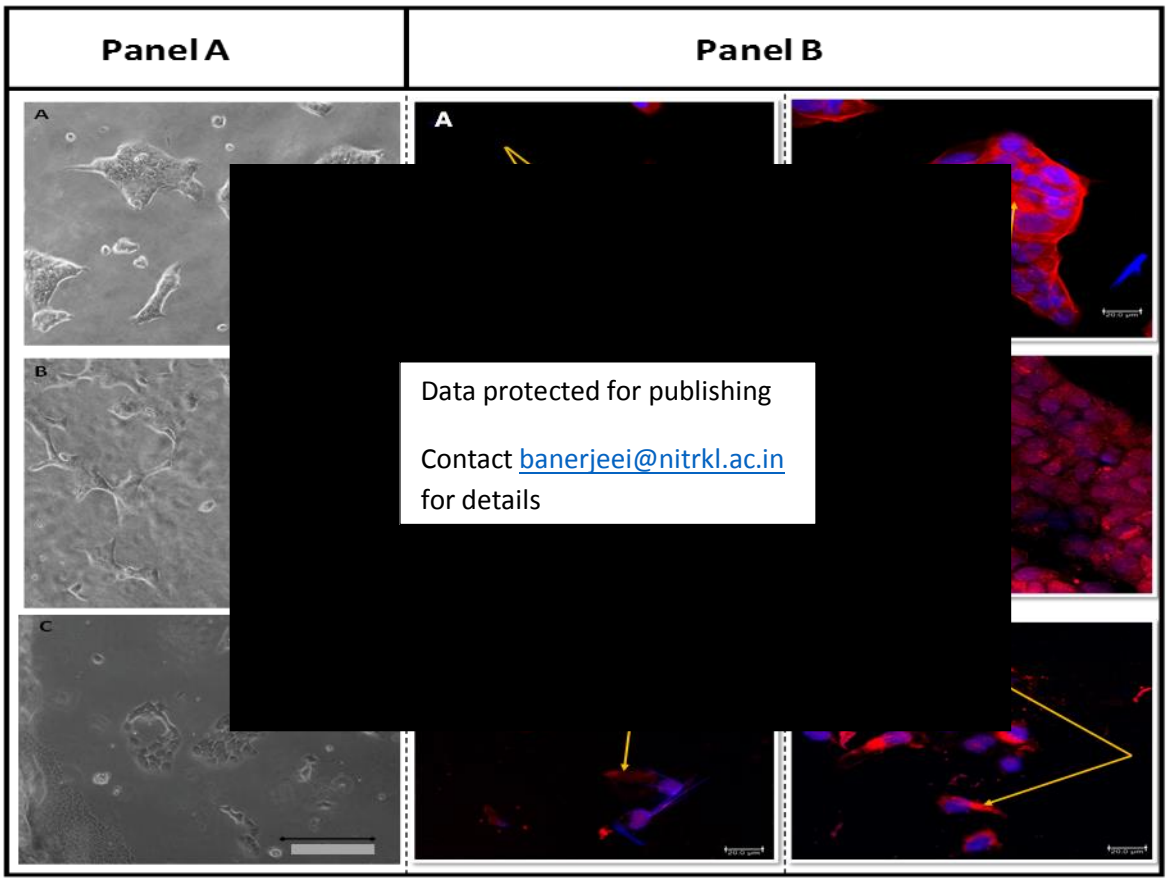


Figure 6: Panel A: Phase-contrast Microscopy [10x] for varying flow rates (shear stress), Panel B: Confocal Microscopy [40x] of actin and nucleus for varying flow rates (shear stress). A. 0.6ml/hr (0.06dyne/cm^2), B. 6ml/hr (0.6dyne/cm^2), C. 60ml/hr (6dyne/cm^2).

Later the flow time optimization was performed for the flow rate of 0.6ml/hr by a live imaging experiment under phase contrast microscopy for 8 hours. The results showed that starting from 4th to the 6th hours of flow exposure the HaCaT cells showed good spreading and colony morphology compared to static control with no flow. Also, interestingly on further exposure of cells to flow for 7 and 8 hours had no significant change on prior 6th hour spreading of cells (Figure 7A). Later the snapshots was taken for every 15 minutes of the 8 hour video and cellular area was calculated using ImageJ and plotting the area against time showed a saturation in spreading from 6th to 8th hour of flow exposure (figure 7B) Therefore the study of

Shear flow as mechanoregulator on HaCaT skin keratinocytes was optimized and confirmed to expose at 0.06dynes/cm^2 for the time period of 6 hours for complete study.

Figure 7: A: Snapshot at different time interval of live video captured by HaCaT cells exposed to 0.6ml/hr flow for 8 hours, B. Cellular spreading kinetics plot for 8 hours

3.4 cytoskeletal and nuclear reorganizations due to flow:

A number of studies has reported that the shear stress induced by fluid flow has effects in cytoskeletal reorganization in various type of cells including endothelial cell, bone cells, Skeletal muscle cell, fibroblast cells, etc. [51-53]. The vital cytoskeletal proteins of the cell include intermediate filaments and actin fibers. It is usually well-thought-out that the cellular cytoskeleton endure reorganization in order to repel the stress condition. Our results show that on exposure to shear stress, the actin microfilaments shows a bridge formation between cell-cell junctions. However, on increasing the rate of flow (shear stress of 6 dyne/cm$^{2)}$ resulted in the disruption of actin fibers and thus the cellular damage (Figure 6). It is vital to mention while static conditions the actin cytoskeleton is distributed unsystematically in the form of a mesh. However, under stressed condition the actin was more intense on the cellular outer boundaries with the formation of dense and concentrated stress fibers at junctional points. An acute analysis of cellular area and the nuclear area was done, and its respective elongation factor was also calculated. The results obtained suggest that on exposure to the fluid flow induced shear stress of 0.06 dyne/cm^2, the cellular area and nuclear area increased significantly.

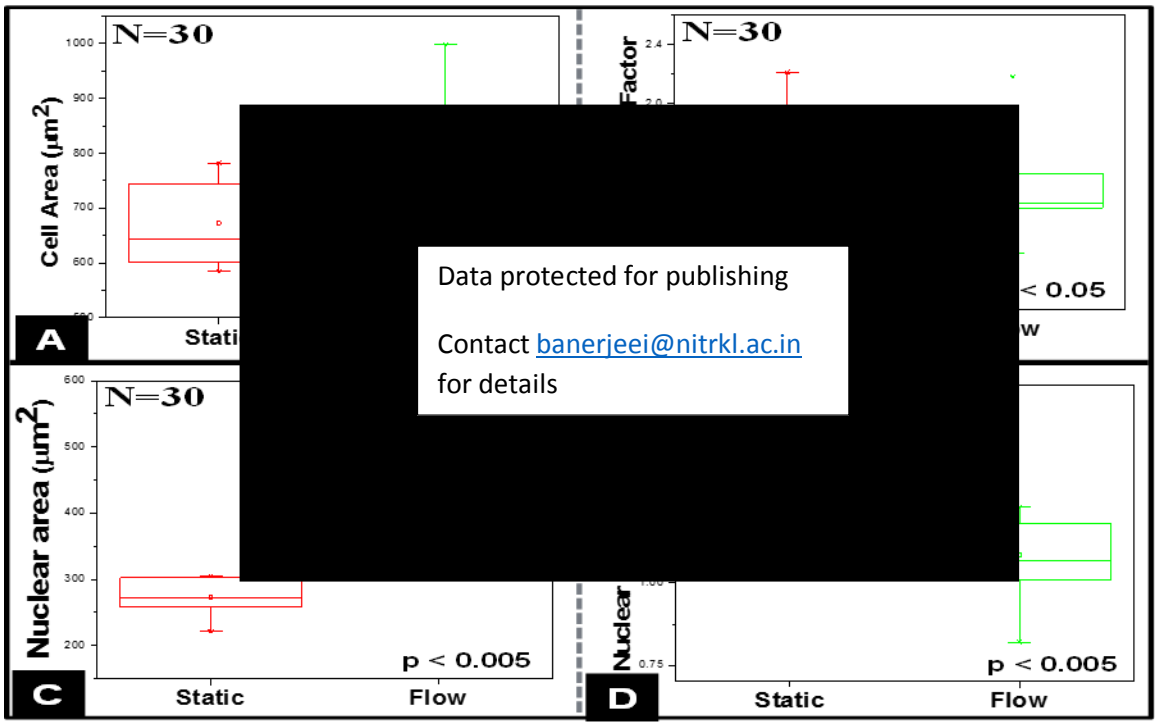


Figure 8: A: Cellular area in static and flow condition, B. Cell elongation factor in static and flow condition, C. Nuclear area in static and flow condition, D. Nuclear elongation factor in static and flow condition.

Also, the increase in fluid induced shear stress also resulted in an increase of cellular elongation. Fascinatingly, exposing the cells to fluid induced shear stress of 0.06 dyne/cm^2 showed an increase of the cellular and nuclear area and also decrease in the nuclear and cellular elongation. It is vital to report that under a shear stress condition of 0.06dyne/cm^2, a 1.34 folds enhancement in the spreading of cells. This clearly deciphers that a lower rate of fluid induced shear stress (0.06dyne/cm^2), exhibit a relevant influence on the HaCaT skin keratinocytes cells via lower elongation and greater spreading.

3.5 Fluid flow induced modulation of HaCaT cell adhesion and respective expression profile of FAK:

The surface receptor and downstream signal transducer integrin are mainly involved in the cell receptor and substrate ligand adhesion and is also known as a vital mechanotransducer [22]. It recruits a variety of adhesion-associated cytoplasmic proteins to form focal adhesion kinase complexes. These focal adhesion complexes have both biochemical and structural function, so integrin receptors are known to play a critical role in mechanotransduction events[54]. The adhesion associated proteins include p130Cas, vinculin,

talin, and focal adhesion kinase (FAK). The changes in the conformation of integrin lead to activation of adhesion associated kinase proteins resulting to changes in localization and expression of cytoskeletal protein [55]. Amongst all, Focal adhesion kinases (FAK's) are well-thought-out to be critical for integrin mediated signaling pathways which their by activates Ras/Raf/MEK/Erk1/2 and PI3K/Akt signaling cascades.

Many researchers have published that FAK has a direct effect on the cellular spreading and adhesion [56]. Our study shows that due to the influence od fluid induced shear stress on HaCaT cells the FAK activation was found to be enhanced in HaCaT skin cell line and along the direction of shear flow the expression of FAK was prominent showing a stress induced adhesive hold of cells to the base, also confirms an additive activation of integrin receptors due to shear force on cells, this may be due to flow induced conformational changes in the extracellular domain of integrin which will further enhance the activation of cytoplasmic events (figure 9A). Also, this data is similar to the reports on endothelial cells under shear stress which align themselves in flow direction and phosphorylation of focal adhesion proteins.

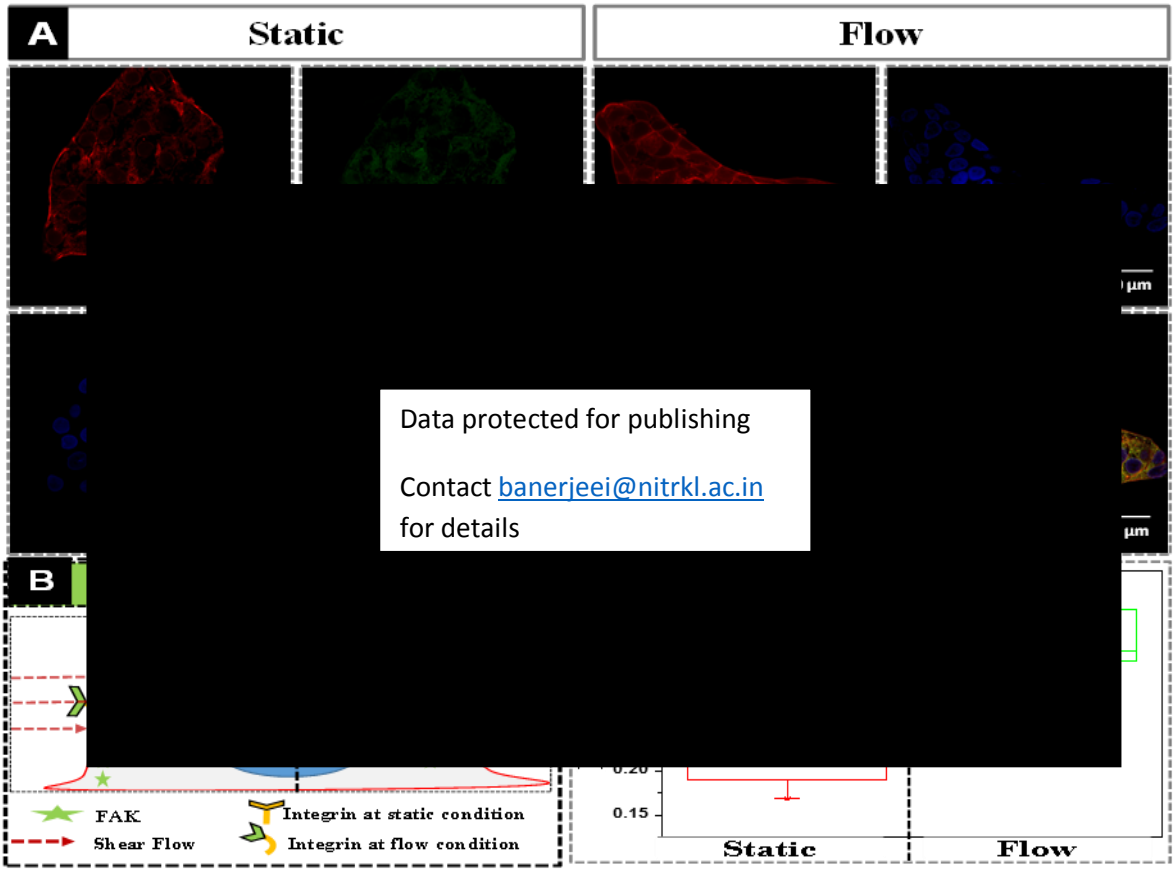


Figure 9: A: Confocal microscopy [60x] images showing the FAK expression, B. Schematics representing the FAK expression due mechanistic activation of integrin due to flow, C. ICQ plots representing Actin/FAK co-localization.

The ICQ plots showed more co-localized expression of FAK showing an effective adhesion kinase activation in HaCaT cells due to flow induce shear stress of 0.06dyene/cm^2 for 6 hours of flow exposure. As a supporting data from the lab unpublished work, we also have the proof for enhanced activation of Erk1/2, which is a downstream effector of FAK. The increased activation of ERK1/2 now can be defined clearly as, may be a consequence of greater activation of FAK.Erk 1/2 which a major regulator for cell spreading [57], in this study observation like enhanced spreading coupled with increased FAK and ERK1/2 expressions delineates the cell signalling track of the HaCaT cells that is involved in integrin mediated mechanotransduction of the shear stress induced by fluid flow and their role in HaCaT cell spreading.

3.6 Flow induced nuclear mechanotransduction and respective expression profile of lamin:

Physical connections between the nuclear envelope and the cytoskeleton provide a mechanism to transmit cytoskeletal and extracellular forces to the nucleus that is critical for nuclear mechanotransduction. The flow induced cytoskeletal organization observed in studies was analyzed by studying various cytoplasmic cell signaling protein molecules. The nuclear mechanotransduction which is an emerging topic of study many researches in resent has shown the importance of lamin as a nuclear mechanotransducer. Also, lamin proteins are composed of different types out of which Lamin AC is considered as a vital maker protein involved in mechanotransduction of cytoskeleton into nucleus. To support the observation of nuclear organization and nuclear elongation due to fluid flow induced shear stress the lamin expression was studied. The nucleus is the stiffest and largest organelle and is exposed to mechanical forces which are transmitted through the cytoskeleton from outside the cell and also from the force generated by within the cell [58]. It was observed in this experiment a keen and clear enhancement in the expression profile of lamin due to flow. All the expressed lamin protein was completely co-localized in the nucleus of HaCaT cells, at the same time the expression of lamin was almost absent in static control without flow (figure 10A). Also, Lamins are the main components of the nuclear lamina but also form stable structures in the nuclear interior. Lamins regulate and support protein complexes involved in DNA replication, gene expression, repair, and transcription, aging and nuclear positioning [59]. So enhancement in lamin expression confirms nuclear mechanotransduction in the HaCaT cells exposed to 0.06dyene/cm^2 shear force induced by fluid flow for 6 hours and gives an idea further to study the lamin mediated nuclear expression in future.

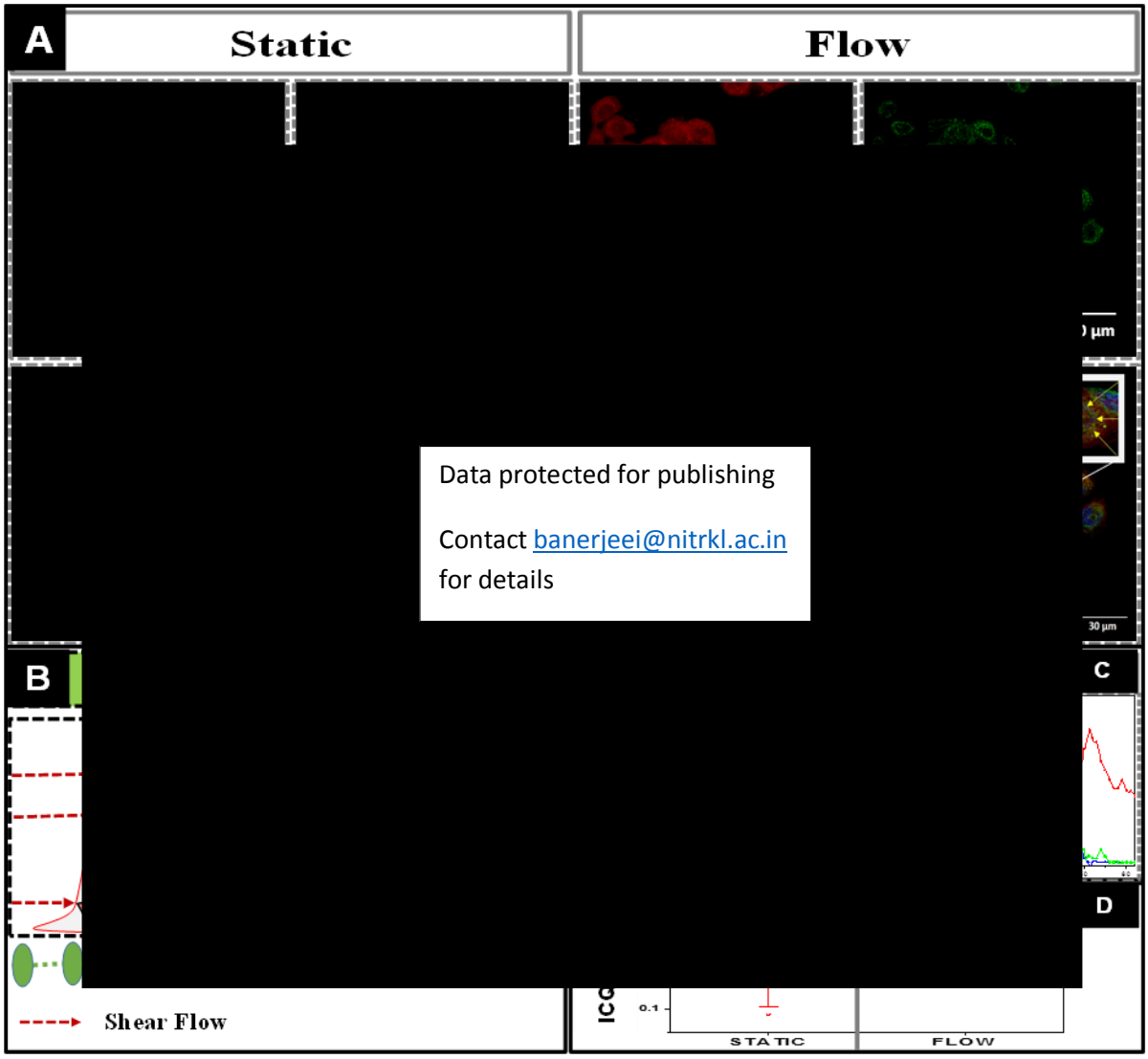


Figure 10: A: Confocal microscopy [60x] images showing the Lamin expression, B. Schematics representing the Lamin expression due transduction of mechanical stimuli through cell cytoskeleton to nuclear membrane, C. Intensity overlap plot of actin nucleus and lamin, D. ICQ plots representing Nucleus/lamin co-localization.

The intensity overlap plot along the cell major axis of actin/nucleus/lamin showed a peak of lamin overlapping in both boundary endings of the nucleus. This overlap was prominent and visible only in flow induce system whereas the static control had nearly no lamin expression. This clearly confirms a direct fluid induced shear stress transduced as mechanical stimuli along the cytoskeleton towards the nuclear membrane. Also, the ICQ plots of nucleus/lamin showed an increased localization of lamin to the periphery of the nucleus due to fluid induced shear stress of 0.06dyene/cm^2 on HaCaT cells for 6 hours.

3.7 Flow induced modulation of tight junction and respective expression profile of E-Cadherin, N-Cadherin and ZO1:

In vertebrate simple epithelial cells tight junctions is identified as an intercellular adhesion that have been thought to act as a primary barrier for the solutes to diffuse through the paracellular route and these junctions occur in the most apical region of the lateral membranes. Tight junction based sealing between cells is consequential for the compartmentalization of the body [60]. To confirm the quality and effectiveness of tight junction, it is vital to study the expression and localization of cadherin and tight junctional protein like ZO1. The E-cadherin expression is reported to be the vital regulator of quality tight junction formation *in vivo* also it serves as an epidermal barrier. Improper co-localization of key tight junction proteins were observed in absence of the E-cadherin and that leads to permeable tight junctions with altered resistance of epidermis and so it is considered in tight junction formation E-cadherin has a major role in signalling rather contact formation [61]. In this study an enhanced E-Cadherin was obtained due to flow (figure 11A) and it was found in comparison to static control there was a 4.02 fold increase in expression of E-Cadherin (figure 11B) due to flow induced shear stress on HaCaT cells.

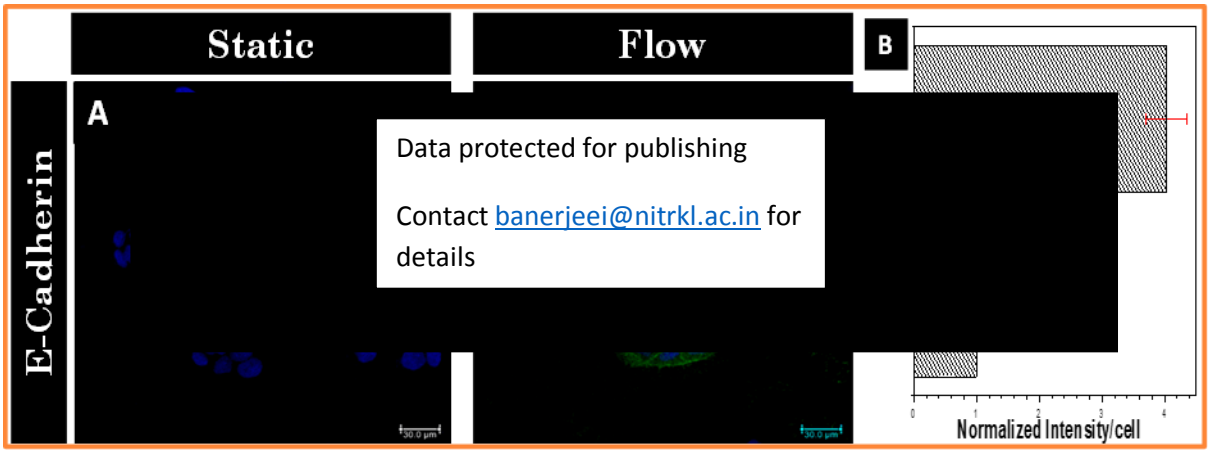


Figure 11: A: Confocal microscopy [60x] images showing the E-Cadherin expression, B. Histogram plot representing the normalized E-Cadherin expression per cell.

In other hand the studies about Cancer metastasis have shown that the lowering expression of E-cadherin and gain in N-cadherin expression will facilitate epithelial to mesenchymal transition [62] demonstrating that the loosening of cells from its colonial junction points is due to lowered E-cadherin expression. In our study we obtained a good actin stress fibre formation at 0.06dynes/cm^2 for 6 hours flow exposure and respective E-cadherin expression at this condition was more expressive when compared to static control without flow

and also it was observed the E-cadherin was mostly localized on to actin stress fibres confirming a quality tight junction formation on the other hand while studying the N-cadherin expression there was no visual change in expression due to induction of flow in system (figure 12A). Compared to static control without flow the expression of N-cadherin was almost same and very poorly localized in comparison with well-formed actin stress fibres, the ICQ plots showed a significant decrease in expression of Actin/N-Cadherin localization (figure 12B). This makes a clear statement that due to shear flow the HaCaT cells forms an excellent quality actin stress fibers. Also enhanced localized expression of E-cadherin reveals the effectiveness of tight junctions formed due to the low shear flow of 0.06dynes/cm^2.

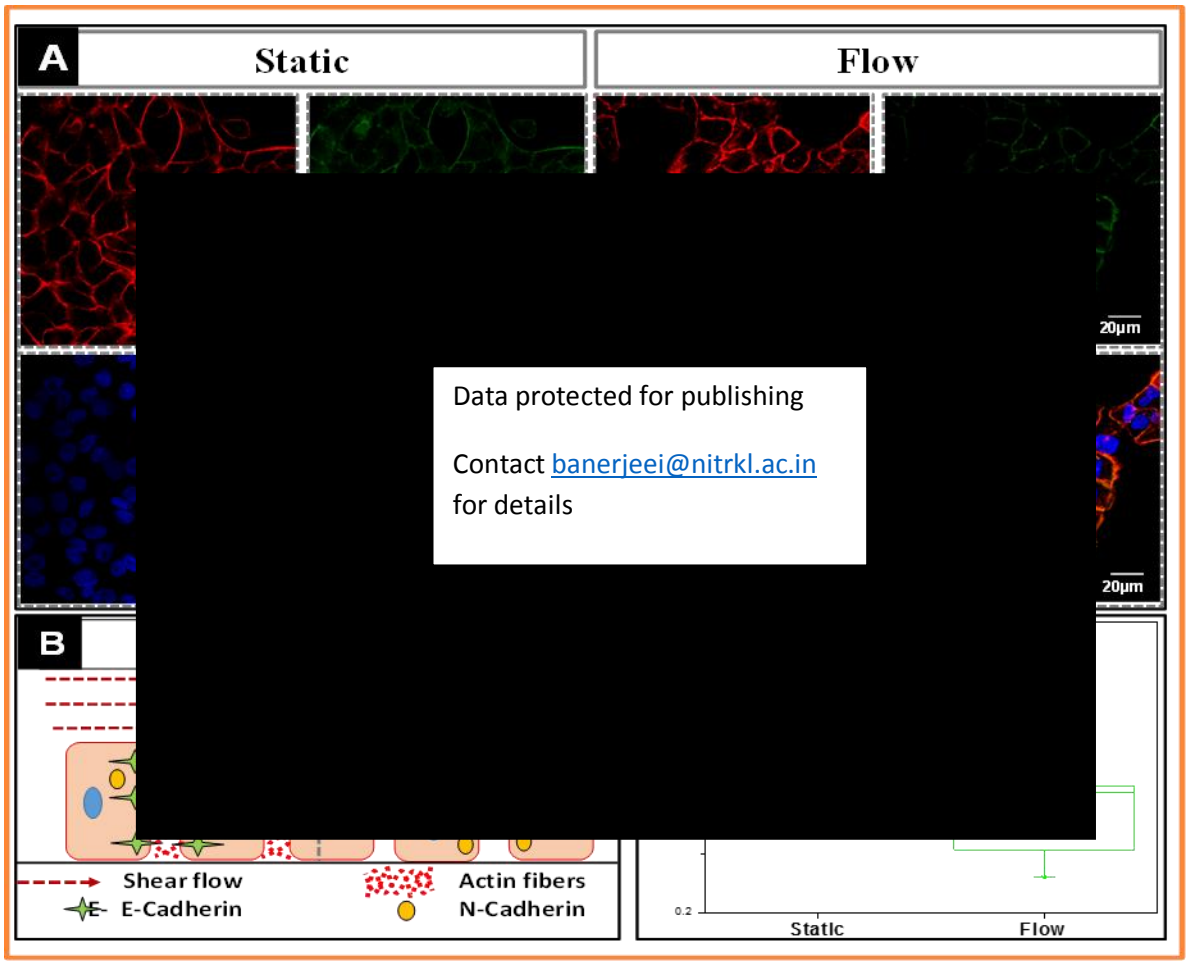


Figure 12: A: Confocal microscopy [60x] images showing the N-Cadherin expression, B. Schematics representing the N-Cadherin/E-Cadherin expression and actin fiber formation due to fluid induced shear stress on HaCaT cells, C. ICQ plots representing Actin/N-Cadherin co-localization.

Another interesting evidence by observation of N-cadherin expression which was similar in both static and flow conditions unveil that cells when exposed to shear flow is not favoring the loosening of formed tight junctions. This clearly gives a view that the HaCaT cell under the shear flow of 0.06dynes/cm^2 for 6 hours of exposure forms a well-defined tight junction complexes and cells strictly tends to stay in their colony morphology. In a study about *Zonula Occludens* (tight junction), it's reported that ZO-1 as the pervasive protein of the tight junction formation in the epithelial system of mammals. Ultrastructural co-localization of ZO-1 is essential to identify positively it as component with a good tight junction [63]. In our results, we found a huge expressive and good actin localized ZO-1 in the flow induced system comparative with that of static no flow system (figure 13A). And these results were visually promising for the effective formation of tight junction statistical significance of the microscopy data obtained was interpreted using MBFimageJ to quantitatively validate the results obtained. The normalized expression/cell histogram plot showed a 2.8 fold increase in expression of ZO-1 tight junction proteins (figure 13B).

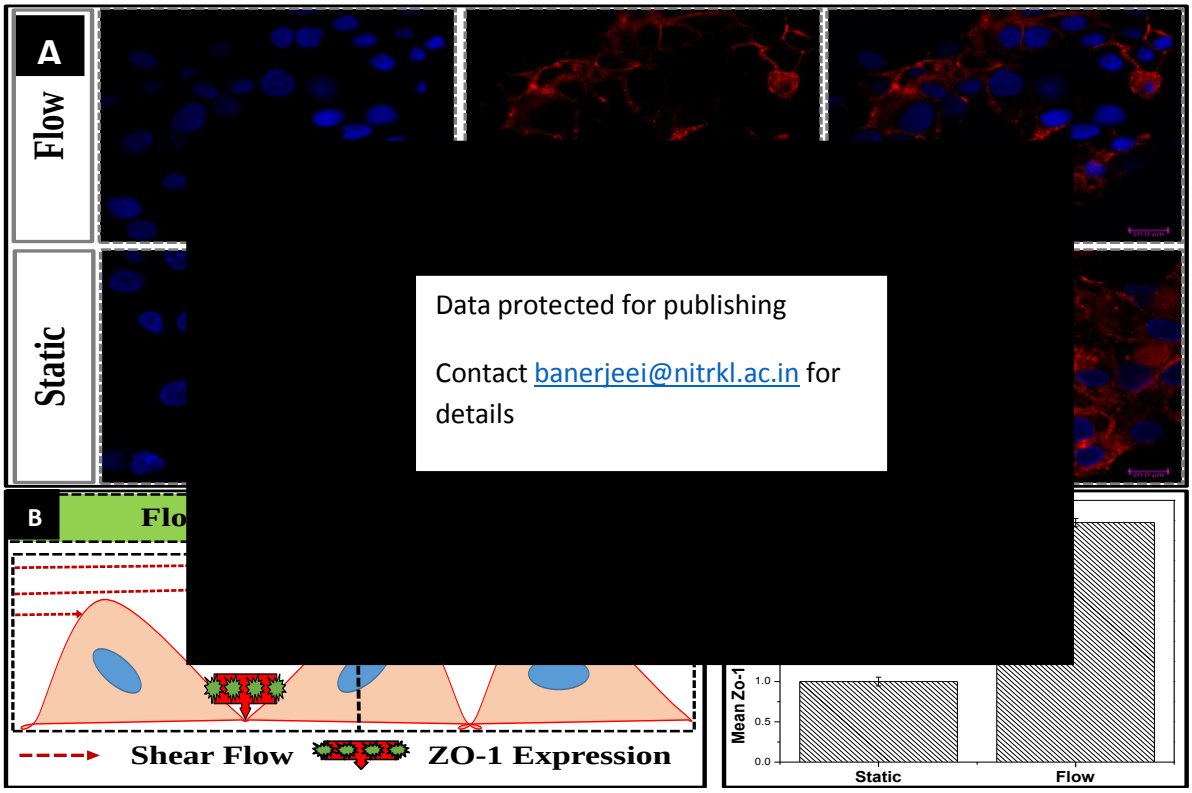


Figure 13: A: Confocal microscopy [60x] images showing the ZO-1 expression, B. Schematics representing the ZO-1 expression in the tight junctional points due to fluid induced shear stress on HaCaT cells, C. Histogram plot of mean ZO-1 normalized expression/cell.

To delineate clearly the tight junctional protein ZO-1 and E-Cadherin profile due to flow a dual staining protocol of immunocytochemistry was followed. In this experiment, the cells were stained to two different antibodies ZO-1 and E-Cadherin respectively having a different binding domain to rabbit (red) and mouse (green) secondary antibodies. The results visually showed a very prominent expression of tight junctional protein ZO-1 and along the tight junctional complex a defined E-Cadherin expression was also observed in flow induced system whereas the static control without flow dint show major expression for both antibodies (figure 14A). The statistical box plot of ICQ values showed a significant co-localized expression of ZO-1/E-Cadherin (figure 14B).

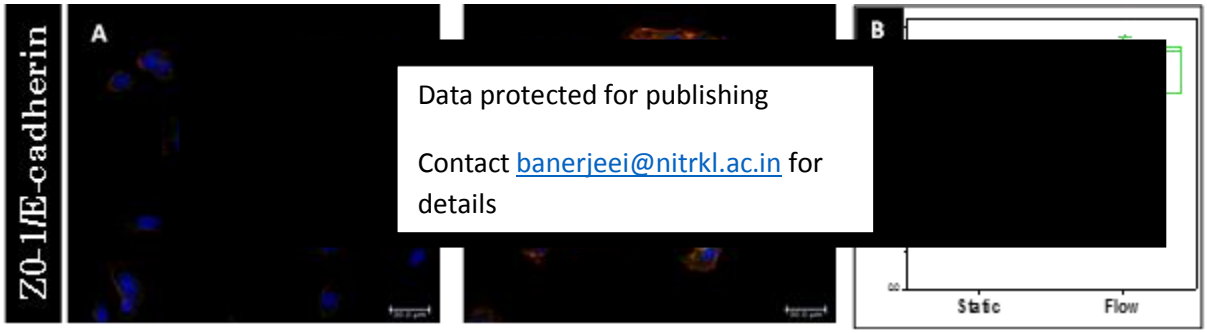


Figure 14: A: Confocal microscopy [60x] images showing the ZO-1/E-Cadherin expression, B. Box plot for ICQ values of ZO-1/E-Cadherin colocalization.

3.8 FAK profiling in presence of blockers to check the efficiency of blocker and its function in mechanotransduction:

Researchers to track a signaling pathways have always used a method of blocking a downstream and upstream effectors to confirm the signaling path [64]. To improve the study and delineating the mechanotransduction pathway blocked coupled studies was performed. The major known mechanotransducers are microfilaments, motor proteins and cell membrane itself and to confirm their functional loss due to blocking, the FAK was used as a model effector for the study. So the study was done by blocking each of them individually before flow and the expression of FAK was confirmed by confocal studies. The ICQ index of FAK and actin co-localization was taken as the result to confirm the activity of blockers in our flow induced system (figure 15). The FAK profiling showed a very promising data and the importance of microfilaments actin and motor proteins in mechanotransduction. Blocking actin and myosin positively reduced the expression of FAK and disrupting lipid rafts caused a little effect when compared to the microfilaments and motor proteins. Also interestingly when MEK downstream

effector of FAK was blocked the expression of FAK remained nearly the same comparative to the normal flow control and this suggests that the shear induced mechanotransduction takes place in HaCaT cells majorly through actin/ myosin-II mediated path and the mechanical stimulus mediated expression is independent of MEK and rock pathway.

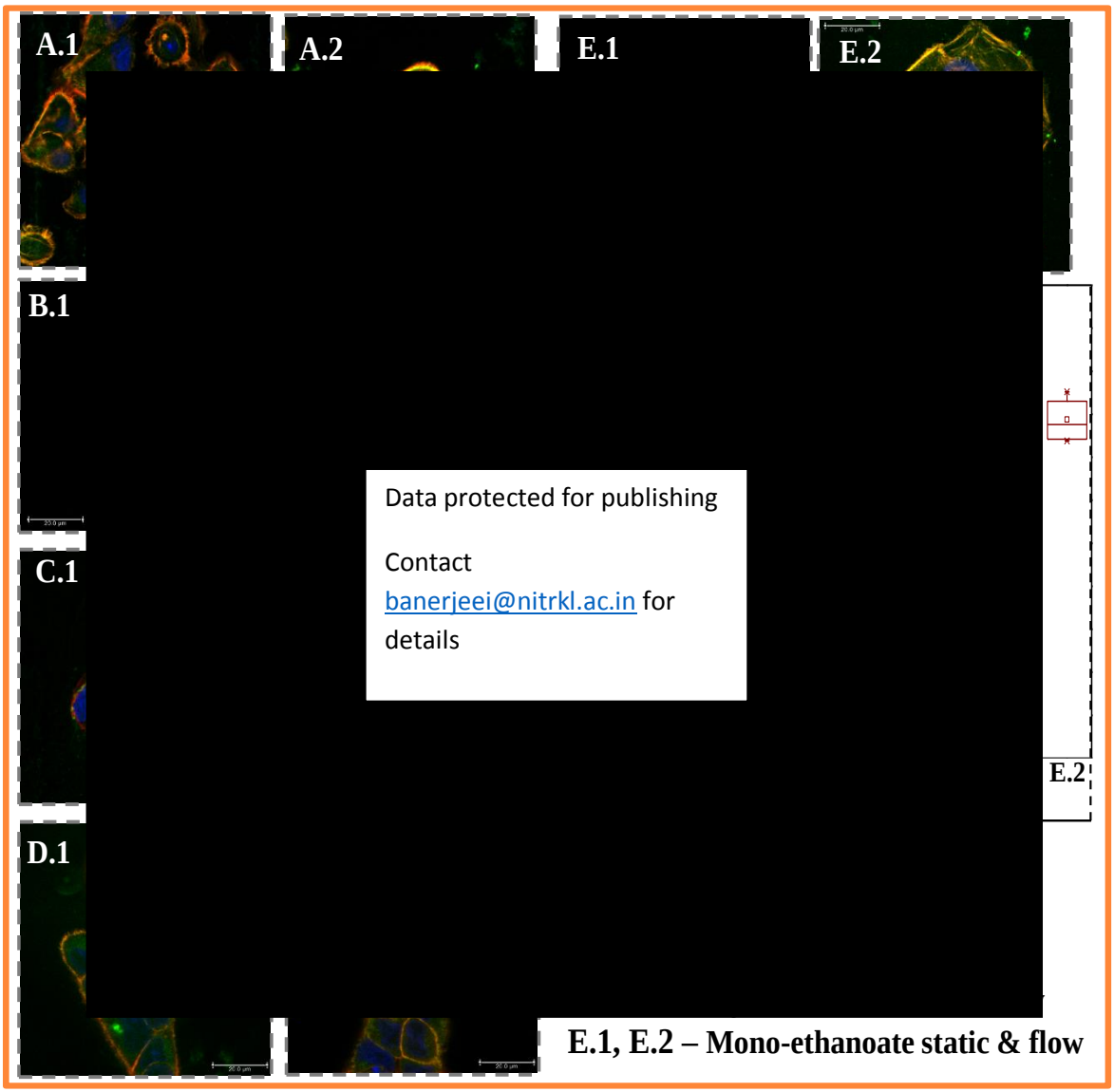


Figure 15: A: Confocal microscopy [60x] images showing the FAK expression in both flow and static control after treatment with blockers with their respective Actin/FAK co-localization ICQ box plot.

CHAPTER 4

CONCLUSIONS

4. Conclusions

In the present study, we have demonstrated the fluid flow induced shear stress is a mechanoregulator of the epidermal keratinocytes. We report that flow induced shear stress could effectively regulate the skin keratinocyte behaviour recognized in terms of its morphology, cytoskeletal reorganization, nuclear reorganizations and protein expression. We therefore propose that the mechanotransduction path involved in cellular response to shear stress follow majorly an actin coupled track, minor role of myosin motor protein is also involved and also has a vital role in mechanistic activation of integrin like surface receptor. The expression of lamin due to flow induced shear stress also confirms the nuclear mechanotransduction and path yet remains unexplored. The main problem of focus in this study was to enhance the cell sheet formation by flow induce shear stress on HaCaT cells and the observations of increasing E-Cadherin and ZO-1 expression along with the decreased expression of N-Cadherin clearly delineates that the Flow induced shear stress very much favors the epithelial characteristics and thereby confirming the effectiveness of cell sheet engineering. The most interesting observations of chemotaxis during live imaging and the characteristics of cell migration were mostly in the direction opposing flow and clear lamellapodia movement of single cells towards the nearby colonies during flow was observed. This opens a new gate for exploration yet to be made in our laboratory in fore coming days.

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